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Micrografting of ASBVd-infected Avocado (*Persea americana*) plants

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Abstract

Shoot tips (meristem plus 2–3 leaf primordia) from *in vitro*-germinated avocado seedlings of 2 ASBVd-infected cultivars were micrografted *in vitro* onto decapitated seedlings from 2 ASBVd-free cultivars, and plants were recovered. Shoot tips consisted of two different sizes, i.e., <0.5 mm long and >0.5 mm but <1 mm long. The recovered plants were indexed for ASBVd using RT-PCR. More plants (58.8%) were recovered from scions >0.5 mm than from those that were <0.5 mm (10.3%). RT-PCR demonstrated that ASBVd replicated in all micrografts from infected sources irrespective of the scion size, while no ASBVd was detected in micrografts from plants that tested negative. ASBVd therefore cannot be eliminated by *in vitro* micrografting.

Introduction

Avocado sunblotch viroid (ASBVd) is a member of the *Avsunviroidae* family of viroids and causes sunblotch disease of avocado (*Persea americana* Mill.) (Palukaitis et al., 1979). ASBVd affects fruits, leaves and possibly roots, and the levels of infection in the plant have been observed to vary according to the tissue of origin and environmental conditions (Schnell et al., 1997); asymptomatic trees can occur (Semancik and Szychowsky, 1994). This has been interpreted as being due to unequal distribution of the viroid, lack of efficiency of the indexing technique and possible silencing mechanism of the host on the viroid (Olano et al., 2003). Transmission of ASBVd can occur via seeds, vegetative planting material, pollen and contaminated tools (Desjardins et al., 1980). The only effective control measure is eradication of affected plants. Based on indexing procedures of low sensitivity, micrografting and meristem tip culture of viroid-infected material have been reported to eliminate viroids of the *Pospiviroidae* family in citrus, (Murashige et al., 1972) and grapevine (Duran-Vila et al., 1988). Micrografting was therefore studied as a means of eliminating ASBVd from infected avocado material.

Materials and methods

Plant material

Avocado (*Persea americana* Mill.) plant material was obtained from the National Germplasm Repository of the USDA-SHRS, Miami FL. Seedlings for rootstocks were obtained from ASBVd- free 'Simmonds' (Miami# 7831) and 'Wilson Popenoe' (Miami# 20032). Scion-donor seedlings were obtained from ASBVd-infected 'Donaldson' (Miami# 20024) and 'Vero Beach' GRD (Miami# 20536).

Seedling rootstock preparation

Seeds were removed from mature fruits; the seed coats were removed and surface-disinfested for 20 min in 1.5% (v/v) sodium hypochlorite solution containing 5 drops of Tween-20® L^{-1} and rinsed with three changes of sterile deionized water. Under axenic conditions in a laminar flow hood, each surface-disinfested seed was bisected longitudinally and the zygotic embryo was removed with an approx. 1 cm^3 of cotyledon (Pliego-Alfaro and Murashige, 1987). A single embryo was cultured in each Petri dish (100 × 20 mm) containing 20 ml of semi solid avocado germination medium (AGM) (Pliego-Alfaro and Murashige, 1987). AGM consisted of Murashige and Skoog (1962) (MS) medium supplemented with (in mg l^{-1}): sucrose (30,000), myo-inositol (100), thiamine HC1 (0.4) and TC agar (8000) (Carolina Biological). Petri dishes were sealed with Parafilm® and stored in translucent plastic boxes. There were 63 'Vero Beach' GRD embryos, 90 'Simmonds' embryos, 135 'Donaldson' embryos and 120 'Wilson Popenoe' embryos. The percentage of germination was calculated 1–2 weeks after inoculation.

Micrografting

Prior to micrografting, all leaves except for 2–3 of the smallest leaf primordia were removed from the scion donor plants. For the rootstocks, the leaves were removed from 2–3 cm long seedlings and all lateral shoots were removed, leaving a single shoot. Using a sterile razor blade, the rootstock seedling was decapitated approx. 1 cm above the medium surface. A shoot-tip (meristem plus 2–3 leaf primordia) was excised with a different scalpel from the scion donor and placed on the decapitated seedling. Since the diameter of the rootstock was greater than that of the scion, the latter was placed off-center and in contact with the vascular ring (Navarro et al., 1975). In order to prevent cross-contamination between each cut, the scalpel was dipped in a 1.25% (v/v) sodium hypochlorite solution, air-dried, dipped in 95% ethanol and flamed. To prevent dehydration of the micrografted shoot tips, a 2 mm^3 cube of semi solid AGM was placed next to the graft union. Thereafter, the Petri dish was covered and sealed with Parafilm®. All manipulations were performed within a laminar flow hood and using a dissecting microscope. Each micrograft was marked with the same number of the scion-donor seedling for further indexing correlation. There were 136 micro graftings: 40 of 'Vero Beach' GRD on 'Simmonds' and 96 of 'Donaldson' on 'Wilson Popenoe'. Two different sizes of scion were evaluated in each micrografted pair: half of the experimental units were performed with scions < 0.5 mm long and the other half with scions > 0.5 mm but never >1 mm long. The number of successful micrografts was recorded 5–6 weeks after micrografting and the percentages of success with respect to genotype and scion size were recorded.

Micrografted plants were transferred into 150 × 25 mm borosilicate test tubes containing 25 ml sterile AGM. The test tubes were capped with Kaputs®, and sealed with Nescofilm®. The surviving micrografts were transferred monthly to fresh medium of the same formulation. After three consecutive subcultures, the micrografts were transferred into 266 ml Qorpak® clear glass bottles containing 40 ml of AGM supplemented with 100 mg l^{-1} activated charcoal (Sigma). The bottles were capped with sterile Petri dish covers and sealed with Nescofilm. Lateral shoots that developed from the rootstock were removed. Approximately 15–20 ml of warm AGM was added to the top of older medium at 20-day intervals.

Medium sterilization and culture conditions

The medium was sterilized by autoclaving at 121 °C and 1.1 kg cm^{-2} for 15 min. Cultures were incubated at 25 °C with a 16-h photoperiod provided by cool white fluorescent tubes at 40–50 μmol m^{-2} s^{-1}.

RT-PCR

Plants were indexed for ASBVd infection using RT-PCR (Schnell et al., 1997) 6–7 months after micrografting. Floral tissue from ASBVd-infected 'Vero Beach' SE2 (Miami # 20535) and leaf tissue from ASBVd-negatively indexed 'Simmonds' were used as positive and negative controls, respectively. Approximately 100 mg of tissue was ground to a fine powder using liquid nitrogen in a sterile ceramic mortar; during grinding and while the tissue was still frozen, 1 ml of RNA extraction buffer (Ainsworth, 1995) was added. RNA extraction and cDNA synthesis were according to Schnell et al. (1997). At the end of extraction, the dried pellet was dissolved in 20 μl DEPC-treated water and

RNA was quantified using a GeneQuant (Amersham Pharmacia). Annealing of the primer to the RNA template was performed using 8–10 μl of a sample containing various concentrations of isolated RNA, 3 μl 5X first strand buffer [50 mM Tris HC1 (pH 8.3), 75 mM KC1, 3 mM $MgCl_2$ (Gibco BRL), 0.1 M DTT (Gibco BRL)] and 0.5 μg forward primer (5′-AAGTCGAAACTCAGAGTCGG-3) complementary to nucleotides 68–87 of ASBVd wild type J02020 in the upper conserved region (Bar-Joseph et al., 1985).

First strand cDNA amplifications were performed using 5′-end labeled (6-FAM) forward primer 5′-AAGTCGAAACTCAGAGTCGG-3′ complementary to nucleotides 68–87 of the J02020 ASBVd molecule and 5′-end labeled (HEX) reverse primer 5′-GTGAGAGAAGGAGGAGT-3′, homologous to ASBVd J02020 nucleotides 88–104 (IDT Co.) (Schnell et al., 1997). Amplified PCR fragments were analyzed using capillary electrophoresis in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using 4% Performance Optimized Polymer (POP 4) (Schnell et al., 2001). Data were collected and analyzed using Gene Scan Software (Applied Biosystems).

Results

Germination occurred within 2 weeks after explanting and the percentages of germination were 87.3%, 91.1%, 91.1% and 92.6% for 'Vero Beach' GRD, 'Simmonds' 'Donaldson' and 'Wilson Popenoe', respectively.

Micrografting

Successfully micrografted scions were green; stem elongation was observed 7–10 days after micrografting (Figure 1). Production of new leaves was observed 2–3 weeks after micrografting. Forty-seven (34.56%) plants were recovered from 136 performed micrografts: 10 were micrografts of 'Vero Beach' GRD on 'Simmonds' and 37 were 'Donaldson' on 'Wilson Popenoe' (Table 1). When micrografting was performed using $\geq$0.5 mm long scions, 40 plants were recovered (58.8%), while only 7 (10.3%) plants survived when the scion was <0.5 mm long (Table 1). Among plants recovered from larger scions, 32 were 'Donaldson' on 'Wilson Popenoe', and 5 were 'Vero Beach' GRD on 'Simmonds'. The 7 plants recovered from scions <0.5 mm long included 5 from 'Donaldson' on 'Wilson Popenoe' and 2 from 'Vero Beach' GRD on 'Simmonds' (Table 1).

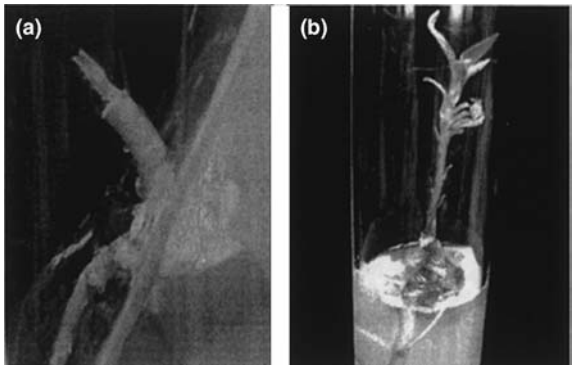

Figure 1. Micrografting showing (a) initiation of scion elongation and (b) growth and development.

RT-PCR

Twenty four micrografted plants were indexed for ASBVd infection. An initial indexing 4 months after micrografting did not detect ASBVd in the micrografts or in the scion-donor plants. Seven months after micrografting, infection was detected in 19 of the 24 indexed individuals. Nineteen micrografted plants showed peaks in electropherograms that migrated at the same size (approx. 250 bp) as the positive control 'Vero Beach' SE2 floral tissue. No peak formation was observed in the

Table 1. Percentages of successful micrografts of avocado

Genotype	Number of micrografts	Scion size				Plants recovered	Success (%)
		<5 mm	Success	>5 mm	Success		
VB GRD /Simmonds	40	20	2	20	8	10	25
Donaldson /W.P	96	48	5	48	32	37	38.54
Total	136	68	7	68	40	47	34.55

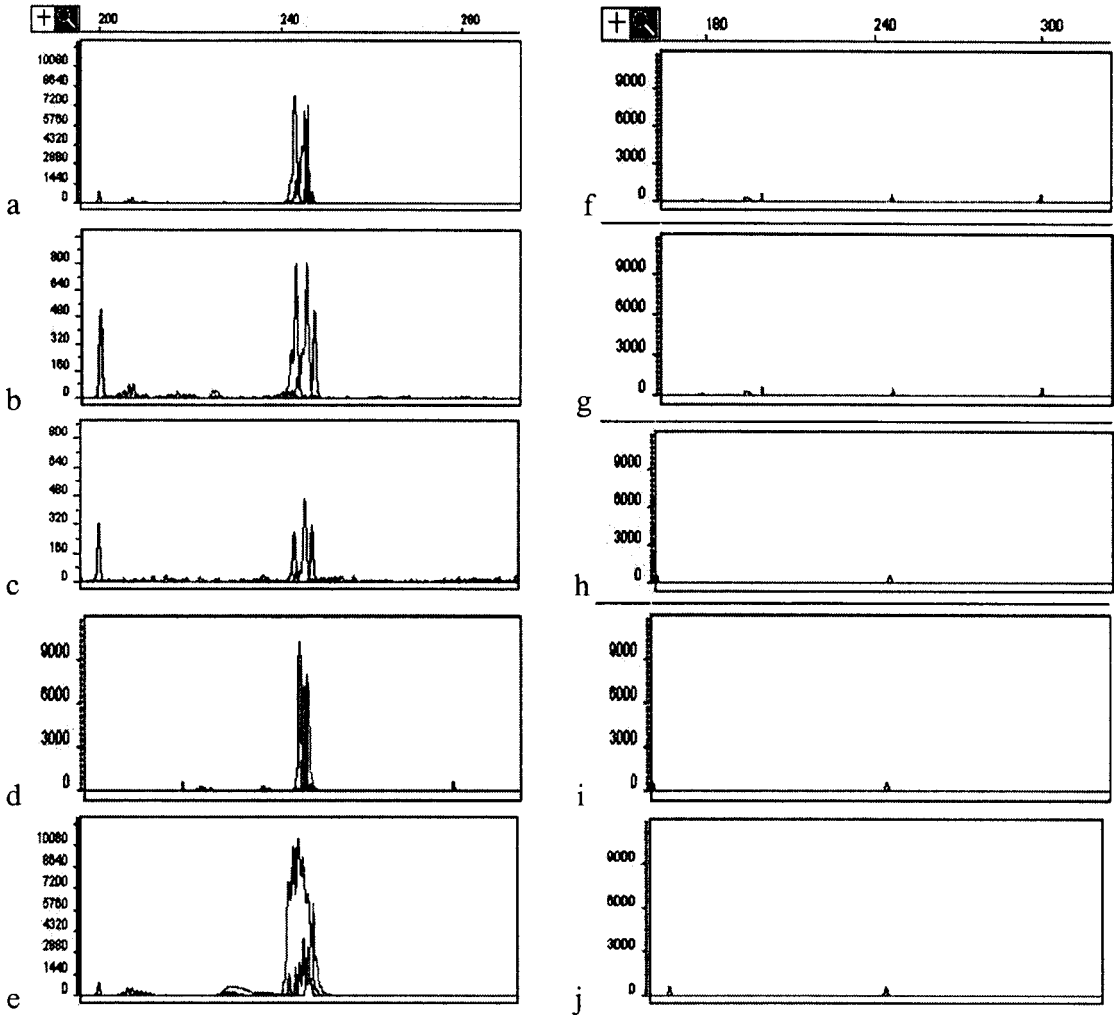

Figure 2. Electropherograms of RT-PCR positive indexed leaves (left) of (a and b): micrograft No. 103 and its respective scion-donor seedling; (c and d): micrograft No. 133, and its respective scion-donor seedling and (e): flower tissue of positive control 'Vero Beach' SE2, and negative indexed leaves (right) of (f and g): micrograft No. 10 and its scion-donor seedling; (h and i): micrograft No. 111 and its scion-donor seedling; and (j): leaf tissue of negative control 'Simmonds'.

negative control 'Simmonds' (Figure 2). Five of the 24 recovered plants did not generate an amplification product (Table 2). RT-PCR of the scion-donor seedlings of the negatively tested micrografting, demonstrated that they were free of ASBVd (Figure 2). Indexing of the scion-donor seedlings of the 19 ASBVd positively seedlings showed amplifications consistent with ASBVd infection (Table 2).

Discussion

Micrografting has been used to recover healthy plants from citrus exocortis viroid (CEVd) infected citrus (Murashige et al., 1972; Navarro et al., 1975; Monteverde et al., 1987). Murashige et al. (1972) reported that successful micrografting of citrus ranges from 5 to 40%, depending on the genotype. Size of the scion and placement on the decapitated rootstock can affect success; Navarro et al. (1975) observed the lowest success (1.8%) with the meristem dome alone and the highest recovery (47.3%) with the apical meristem plus 6 leaf-primordia. Success rates of 14.6% and 34.6% were obtained when the scion consisted of the meristem plus two leaf primordia and the meristem plus four leaf-primordia, respectively. Navarro et al. (1975) observed that up to 50% recovery occurred when the

Table 2. ASBVd in micrografted plants and scion donar plants

Micrograft number	Scion/Rootstock combination	Scion size (mm)	ASBVd Infected	
			Micrograft	Scion-donors
10	VBGRD/Simmonds	>0.5	No	No
12	VBGRD/Simmonds	>0.5	Yes	Yes
15	VBGRD/Simmonds	>0.5	Yes	Yes
25	VBGRD/Simmonds	<0.5	Yes	Yes
35	VBGRD/Simmonds	<0.5	Yes	Yes
52	Donaldson/WP	>0.5	Yes	Yes
57	Donaldson/WP	>0.5	Yes	Yes
66	Donaldson/WP	>0.5	No	No
75	Donaldson/WP	<0.5	Yes	Yes
81	Donaldson/WP	<0.5	Yes	Yes
84	Donaldson/WP	>0.5	Yes	Yes
91	Donaldson/WP	>0.5	Yes	Yes
93	Donaldson/WP	>0.5	Yes	Yes
95	Donaldson/WP	<0.5	No	No
99	Donaldson/WP	>0.5	Yes	Yes
100	Donaldson/WP	>0.5	Yes	Yes
103	Donaldson/WP	>0.5	Yes	Yes
104	Donaldson/WP	<0.5	Yes	Yes
111	Donaldson/WP	>0.5	No	No
118	Donaldson/WP	>0.5	Yes	Yes
125	Donaldson/WP	>0.5	No	No
132	Donaldson/WP	>0.5	Yes	Yes
133	Donaldson/WP	>0.5	Yes	Yes
136	Donaldson/WP	>0.5	Yes	Yes

VBGRD – 'Vero Beach' GRD; WP – 'Wilson Popenoe'.

scion is in contact with the vascular ring. The avocado data show similar recovery with respect to scion size: total recovery 34.35% and 58.8% recovery with larger scions.

CEVd can be eliminated using scions consisting of the meristem dome with up to 6 leaf-primordia (Monteverde et al., 1987). Although larger scion sizes are likely to provide better recovery of plants, the possibilities of recovering healthy material are reduced. Murashige et al. (1972), using scions from diseased citrus plants reported that 4 out of 6 recovered 'Temple' tangor and 1 out of 2 micrografting of 'Eureka' lemon tested negative for citrus exocortis viroid (CEVd) infection using indicator plants. Navarro et al. (1975), using the same bioassay, reported CEVd elimination in all 31 micrografts of 'Cadenera Fina' sweet orange, 5 out of 6 micrografts of 'Temple' tangor and 77 out of 99 micrograftings of 'Robertson' navel oranges. Monteverde et al. (1987) also observed that all six micrografts recovered from CEVd-infected material were free of the viroid using the same indexing method. With the exception of ASSVd elimination from pear plants (Postman and Hadidi, 1995), viroid elimination has been supported by low sensitivity indexing methods. For example, meristem tip culture-derived grapevine plants were found to be infected with low levels of yellow speckle viroid 1, yellow speckle viroid 2 and Australian grapevine viroid that were undetected using polyacrylamide gel electrophoresis (Wan and Symons, 1997). RT-PCR has demonstrated that ASBVd is persistent in micrografted avocado plants in this study that involved more micrografts and a more sensitive indexing procedure.

Studies of *Pospiviroidae* viroids have shown that the apical meristem is not invaded (Momma and Takahashi, 1983). Bonfiglioli et al. (1996) observed that coconut cadang-cadang viroid (CCCVd) and CEVd localize and move long

distances within the plant through the phloem, where they also replicate. Therefore, the isolation and culture of the shoot apical meristem with 1–4 leaf primordia can be used to regenerate viroid-free citrus plants as a result of the absence of vascular tissue in this area (Barba et al., 2003). None of the avocado scions had >4 leaf primordial, and most had either 2 or 3 leaf primordia, which should have been small enough for recovery of ASBVd-free plants Barba et al. (2003).

Unlike *Pospiviroidae* viroids that replicate in the nucleus, the Avsunviroidae viroids replicate in chloroplasts (Flores et al., 1998). How *Avsunviroidae* viroids move within plants is unknown; however, the inability to rescue ASBVd-free material using scion sizes comparable to those used for CEVd elimination may indicate that isolation of non vascularized tissue from avocado shoot apical meristem is more difficult or, that most probably, ASBVd invades the meristem.

Thermotherapy followed by meristem tip culture has been used to eliminate some viroids, e.g., chrysanthemum chlorotic mosaic viroid (Hollings and Stone, 1970) and apple skin scar viroid (ASSVd) from pear (Postman and Hadidi, 1995; Howell et al., 1998). Avocado plants have not been regenerated from meristem tip cultures; temperatures as high as 100 °C for 20 min do not inactivate CEVd (Semancik and Weathers, 1970), while exposure of potato spindle tuber viroid (PSTVd) to temperatures >75 °C causes only some loss of viroid infectivity (Singh and Bagnall, 1968). Attempts to inactivate the viroid in seed, budwood and budlings of avocado by thermotherapy demonstrated that ASBVd could ''withstand any heat treatment regimen that the avocado tissue could'' (Desjardins et al., 1980).

Although micrografting is ineffective for eliminating ASBVd, other strategies ought to be attempted to address this problem, including nucellar culture and transformation with RNase genes that would target the viroid (Sano et al., 1997).

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